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Age and Diet Effects on Fecal Populations and Antibiotic Resistance of a Multi-drug Resistant *Escherichia coli* in Dairy Calves†

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ABSTRACT

Dairy calves are colonized at a very young age by a multi-drug resistant *Escherichia coli* (MDR EC) and research studies indicate that the prevalence is not related to recent use of antimicrobials, but that diet and other environmental factors are likely involved. To further investigate the occurrence of this bacterium, we sampled dairy calves on southwestern United States farms at one week of age through 6 months, and determined not only prevalence, but fecal concentrations of the MDR EC. The influence of feeding pasteurized (PWM) versus non-pasteurized (NPWM) waste milk was examined, and the effect of weaning was investigated. The number of fecal samples positive for MDR EC as well as their populations decreased ($P < 0.01$) with increasing calf age. Slight differences were observed when comparing PWM and NPWM feeding, with MDR EC concentration and prevalence in the latter group generally decreasing at younger ages. No significant differences were observed in the fecal concentrations of MDR EC due to weaning. No clear differences were observed in resistance when comparing calves fed PWM or NPWM. Approximately 41% of the MDR EC isolates collected throughout the study were resistant to 10 or more antibiotics, with two primary phenotypes: ACSSuT and MDR-AmpC. Based on the results herein, it appears that neither pasteurization of the waste milk or weaning, has a significant effect on the prevalence or concentration of MDR EC, and based on the age-associated decline in prevalence, they survive in an immature digestive system with limited bacterial diversity and competition for resources.

Keywords: *E. coli*, multi-drug resistance, dairy calves, age, weaning

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INTRODUCTION

Antimicrobial resistant bacteria are a growing concern worldwide for both veterinary and human medicine (National Academy of Science, 1999). While the increased resistance in pathogenic bacteria is of utmost concern, commensal bacteria can also be highly resistant to a wide variety of antimicrobials and are considered by some as a potential reservoir of resistance elements for the pathogenic strains (Shoemaker *et al*., 2001; Summers, 2002). More specifically, the presence of multi-drug resistant (MDR) non-pathogenic commensal bacteria such as *Escherichia coli* on dairy farms could theoretically provide a pool of transferable resistance genes for important pathogens such as *Salmonella* and *E. coli* O157:H7 (Schmieger and Schicklmaier, 1999; Winokur *et al*., 2001; O'Brien 2002; Hoyle *et al*., 2004).

The general consensus is that antimicrobial-resistant bacteria, to include commensals in humans and animals, are produced, maintained and disseminated due to the selection pressure induced by exposure to antimicrobial drugs (van den Bogarrd and Stobberingh, 2000). Research examining *E. coli* in calves reported that exposure to antibiotic in the feed resulted in the development of not only resistance to the fed antibiotic but several other antibiotics as well (Wierup *et al*., 1975). Others have reported that the discontinuation of feeding an antibiotic-medicated milk replacer to dairy calves resulted in an increase in tetracycline susceptibility in *E. coli* and *Salmonella* isolates during the first three months that a non-medicated milk replacer was fed (Kaneene *et al*., 2008). While exposure to antibiotics certainly contributes to resistant bacteria, other non-antibiotic influences have been reported (Sogaard 1973; Smith 1975; Gellin *et al*., 1989; Gilliver *et al*., 1999).

Younger animals generally harbor more resistant enteric flora than older animals (Wierup, 1975; Martel and Coudert, 1993). Pre-weaned calves have been reported with higher MDR levels in enteric flora, possibly a result of increased fecal-oral transmission, higher strain turnover within the gastrointestinal tract, or higher levels of antimicrobial drug use in younger animals (Howe and Linton, 1976; Hinton *et al*., 1985). Mature dairy cows sampled in 21 states and cultured for *E. coli* and *Salmonella* found that the majority of isolates (greater than 80%) were susceptible to all antibiotics examined (Lundin *et al*., 2008). Houser and colleagues (2008) reported that 62% of the *E. coli* isolated from healthy lactating dairy cows were susceptible to all antibiotics examined and 21% were resistant to only one antibiotic, ampicillin. We reported similar results when examining dairy cattle of various ages for MDR *Salmonella* (Edrington *et al*., 2008). In this research we found that young calves, prior to weaning, were more likely to harbor MDR *Salmonella* than all other classes of dairy animals (heifers, lactating and dry cows) examined. The primary exception was cows in the hospital pen, as they also exhibited significant levels of MDR *Salmonella*. We hypothesized that the reasons for the high incidence of MDR *Salmonella* in these two groups was a result of previous antimicrobial treatment, as these two groups of cattle are the most likely to receive antibiotic therapy, and/or due to a disturbed or underdeveloped gastrointestinal microflora. In the case of young calves, their intestinal microflora is developing and changing with the introduction of new feedstuffs, weaning, environmental exposure, and other factors, whereas the cows in the sick pen are generally off-feed resulting in a disturbed microflora vulnerable to competition from new bacterial species.

Several studies have documented the prevalence of a highly resistant *E. coli* in dairy calves; however, the results did not provide for a complete description of the early temporal shifts or compare calves from different geographic regions and management systems (Wierup, 1975; Howe and Linton, 1976; Hinton *et al*., 1984; Khachatryan *et al*., 2004). Interestingly, these MDR *E. coli* do not appear to be specific to a geographic region or management practice, having been reported in Washington (DeFrancesco *et al*., 2004), Pennsylvania (Houser *et al*., 2008), and the SW United States (Edrington, unpublished data). Others have documented that pre-weaned calves had the greatest prevalence of resistant *E. coli*, with levels decreasing with increasing animal age (Khachatryan *et al*., 2004). Healthy dairy calves were reported to

be rapidly colonized by antibiotic-resistant strains of *E. coli* shortly after birth (Donaldson *et al.*, 2006) with the highest prevalence observed in 2-week old calves. Calves were reported to shed MDR bacteria resistant to 9 and 10 antibiotics as early as one day of age (Donaldson *et al.*, 2006) with similar observations reported by others (Orden *et al.*, 2000; Werckenthin *et al.*, 2002). The question then arises: Are the levels of resistance in these calves a result of previous/current antibiotic exposure? Berge and co-workers (2006) reported higher levels of MDR *E. coli* in calves fed antimicrobials compared to those on non-medicated feed. Isolates cultured from older calves not fed antimicrobials (14 and 28 d old), had higher levels of resistance compared to day old animals with 14-day old calves most likely to shed increasingly resistant bacteria (Berge *et al.*, 2006). In contrast to this, others have reported that the maintenance of the *E. coli* SSuT resistance phenotype in dairy calves was due to environmental components independent of antibiotic selection (Khachatryan *et al.*, 2006a). Further research by this same group (Khachatryan *et al.*, 2006b) reported that the antimicrobial resistant genes are not responsible for the greater fitness advantage of antimicrobial-resistant *E. coli* in calves, but that the farm environment and the diet clearly exert critical selective pressures responsible for the maintenance of antimicrobial resistance genes. Others have also reported that housing and dietary changes, occurring at weaning, may affect the prevalence of antibiotic-resistant strains by altering the calf's exposure to other animal stock and bacterial strains that in turn change the *E. coli* composition of their gut microflora (Hoyle *et al.*, 2004). Therefore, the objectives of the current research were to evaluate the effect of age, diet (pasteurized or non-pasteurized waste milk), weaning and farm origin on fecal populations and prevalence of MDR *E. coli* in dairy calves. Antimicrobial susceptibility patterns were also examined.

MATERIALS AND METHODS

Animals and Sample Collection

This research was conducted on several large commercial dairies (greater than 3000 head) in the southwestern United States. Four collections were made for this research project. The first sampled calves on two farms representing six age groups (1 week, 2 weeks, 1, 2, 4 and 6 months of age). Fecal samples (approximately 20 g) were collected from freshly voided, undisturbed fecal pats from 15 animals per age group on each farm (n = 90 samples/farm; 180 total samples). Both farms utilized waste milk to feed the calves prior to weaning, one farm pasteurizing the milk prior to feeding, the other using non-pasteurized waste milk. A second similar collection was made, the only difference being that a different farm utilizing pasteurized waste milk was sampled. A total of 360 samples were collected and cultured for multi-drug resistant *E. coli* (MDR EC). A third collection was made in order to evaluate the influence of weaning on the prevalence of MDR EC in dairy calves and was part of a larger study examining the role of weaning on the prevalence of a number of important bacteria (Edrington *et al.*, 2011). Two groups of calves were utilized, the first weaned at approximately 12 weeks of age (avg. BW = 122 kg) and the second group at approximately 10 weeks of age (were not weighed at weaning; estimated BW = 110 kg). Fecal samples were collected from all calves via rectal palpation on two occasions, two days pre- and again two days post-weaning for bacterial culture described below. The fourth collection sampled newborn calves (1 to 3 days of age) from four different dairies during their first week of arrival at a central calf rearing facility. Rectal fecal samples were collected into sterile palpation sleeves from 38, 45, 69 and 40 calves (n = 192 total samples) representing each of the four farms over a four-week period.

Bacterial Culture and Isolation

All fecal samples were collected into sterile palpation sleeves, placed on ice and shipped to our laboratory in College Station, Texas for processing the day following collection. For culture and quantitation of MDR EC populations, 10 g of fecal material was diluted in 90 mL of tryptic soy broth

and plated on MacConkey's agar containing 32 µg/mL chloramphenicol, using a commercially available spiral plater. Following incubation (24 h, 37° C), colonies exhibiting typical *E. coli* morphology were manually counted to determine colony forming units (CFU)/g feces. This was converted to CFU (log_{10})/g feces for statistical analysis and data presentation below. A portion of the isolates from each collection were confirmed as *E. coli* using the API 20E test kit (BioMerieux, Durham, NC). Isolates were stored as glycerol stocks (10% v/v) in TSB at - 80°C. All media and agar were from Difco Laboratories (Detroit, MI). Reagents and antibiotics were obtained from Sigma Chemical Co. (St. Louis, MO).

Determination of Antimicrobial Susceptibility

Antimicrobial susceptibility was determined using the Sensititre automated antimicrobial susceptibility system according to the manufacturer's directions (Trek Diagnostic Systems, Westlake, OH). Broth microdilution was used according to methods described by the National Committee for Clinical Laboratory Standards (CLSI 2005) using the NARM's panel for gram-negative isolates. Resistance breakpoints were determined using the CLSI (CLSI 2005) interpretive standards unless unavailable, in which case breakpoints in the NARMS 2000 Annual Report (FDA 2000) or those provided by Trek Diagnostic were

Table 1. Fecal prevalence of MDR EC (number and populations) in dairy calves of multiple ages, housed on two commercial dairy farms and feeding pasteurized (PWM) or non-pasteurized (NPWM) waste milk through weaning

	Calf Age					
Item	1 wk	2 wks	1 mo	2 mos	4 mos	6 mos
Collection 1						
Farm A - PWM						
no. positive	15/15	15/15	15/15	15/15	13/15	9/15
CFU(log_{10})/g feces	2.7^{bB}	5.4^{A}	6^{A}	5.2^{aA}	3.7^{aB}	2.4^{B}
Farm B - NPWM						
no. positive	15/15	15/15	15/15	10/15	8/15	4/15
CFU(log_{10})/g feces	5.1^{aA}	5.2^{A}	5.8^{A}	3.1^{bB}	2.2^{bBC}	1.8^{C}
Collection 2						
Farm A - PWM						
no. positive	14/15	15/15	15/15	15/15	15/15	14/15
CFU(log_{10})/g feces	5.3^{B}	6.3^{aA}	5^{B}	5.4^{aB}	5^{aB}	3.8^{aC}
Farm B - NPWM						
no. positive	15/15	15/15	15/15	10/15	14/15	9/15
CFU(log_{10})/g feces	5.4^{AB}	5.9^{bA}	4.9^{B}	2.9^{bC}	2.9^{bC}	2.2^{bC}

^{ab}CFU within collection and age column with different superscripts differ ($P < 0.05$).
^{ABC}CFU within collection and farm row with different superscripts differ ($P < 0.05$).
Culture negative samples assigned value of 1.0.

used. *Escherichia coli* ATCC 25922, *E. coli* ATCC 35218, and *Enterococcus faecalis* ATCC 29212 were used as quality control organisms.

Statistical Analysis

Data were analyzed using SAS Version 8.02 (SAS Inst. Inc., Cary, NC, USA). Quantitative data expressed as CFU (log_{10})/g feces were subjected to analysis of variance appropriate for a completely randomized design. A value of 1.0 was assigned to all negative samples for statistical analysis. Pen prevalence was subjected to Chi-square analysis using the PROC FREQ procedure. Means were considered different at a 5% level of significance.

RESULTS

Influence of Age on Prevalence and Antimicrobial Susceptibility of MDR EC

The prevalence and concentration of MDR EC is presented by age and by farm [feeding pasteurized (PWM) or non-pasteurized waste milk (NPWM)] in Table 1 for the two collections. The number of fecal samples positive for MDR EC decreased with increasing calf age during both collections, with the decrease being more pronounced when comparing the farm feeding NPWM versus the two farms feeding PWM. Fecal concentration of MDR EC likewise decreased ($P < 0.01$) with increasing age on all farms for both collections (Table 1). When comparing type of waste-milk fed, MDR EC concentration decreased more rapidly with increasing age in the farms feeding NPWM (Table 1).

Antimicrobial susceptibility was examined in MDR EC isolates (six isolates/age group/collection; n = 72 total MDR EC isolates). In general, during the first collection, more resistance was observed in the farm using NPWM compared to collection 2, when the opposite trend was observed, therefore the data was pooled across farm and presented by collection date in Table 2. All isolates were resistant to chloramphenicol and tetracycline and all but one were resistant to sulfisoxazole, whereas the majority of the isolates were susceptible to ciprofloxacin and ceftriaxone. The number of isolates resistant to all other antibiotics examined decreased with increasing calf age at each collection time (Table 2).

Multi-drug resistance and resistance phenotypes are presented in Table 3. One isolate was resistant to two antibiotics with all other isolates resistant to four or more antimicrobials. Thirty-eight percent of the isolates were resistant to 10 or more antibiotics, the majority of which were cultured in the November collection. Primary resistance patterns observed were ACSSuT and MDR-AmpC, the first of which was more prevalent in the second collection and the frequency of the MDR-AmpC pattern similar among collections (Table 3).

Influence of Weaning on Prevalence and Antimicrobial Susceptibility of MDR EC

Samples were collected from two groups of calves immediately prior to and following weaning and cultured for MDR EC (Table 4). No significant differences were observed in the fecal concentrations or in the number of MDR EC positive pens in either group or when data was combined across groups. There was a tendency ($P = 0.06$) for fewer MDR EC positive pens in the second group of calves post-weaning.

Twenty MDR EC isolates were examined for antimicrobial susceptibility (five per group pre- and post-weaning). All of the isolates were susceptible to amikacin, ceftriaxone, ciprofloxacin and naladixic acid, and all but one isolate susceptible to amoxicillin/clavulanic acid, cefoxitin and ceftiofur. All isolates were resistant to kanamycin, sulfisoxazole and tetracycline and all but one resistant to chloramphenicol (data not shown). Half of the isolates were resistant to four or five antibiotics and most of the remaining half of the isolates (nine isolates) resistant to six, seven, or eight antibiotics (Table 5). One isolate was resistant to 10 antibiotics. Several patterns of resistance were observed, the most prevalent being ACSSuT. One isolate demonstrated the MDR-AmpC pattern of resistance (Table 5). Weaning did not appear to have any influence on antimicrobial resistance in these isolates.

Table 2. Antimicrobial resistance profiles of MDR EC isolates cultured from fecal samples, by collection, from dairy calves of multiple ages on commercial dairy farms. Data represents the number of isolates resistant to the minimum inhibitory concentration (MIC) listed for each antibiotic

			Calf Age						Combined
Item	MIC	Collection	1 wk	2 wks	1 mo	2 mos	4 mos	6 mos	Ages
No. isolates examined		1	6	6	6	6	6	6	36
		2	6	6	6	6	6	6	36
Antibiotic									
Amikacin	≥ 64	1	2	3	3	2	0	0	10
		2	3	2	1	1	0	0	7
Gentamicin	≥ 16	1	4	5	3	2	1	0	15
		2	5	5	5	4	1	2	22
Kanamycin	≥ 64	1	6	6	6	6	2	3	29
		2	6	6	6	6	5	4	33
Streptomycin	≥ 64	1	6	6	6	4	5	3	30
		2	6	6	6	6	4	5	33
Ceftiofur	≥ 8	1	3	3	3	0	0	0	9
		2	4	3	4	4	1	1	17
Ceftriaxone	≥ 64	1	0	0	0	0	0	0	0
		2	1	0	0	0	0	0	1
Cefoxitin	≥ 32	1	3	3	4	0	0	0	10
		2	4	4	5	4	1	0	18
Ampicillin	≥ 32	1	4	4	4	1	0	1	14
		2	6	6	6	5	1	2	26
Chloramphenicol	≥ 32	1	6	6	6	6	6	6	36
		2	6	6	6	6	6	6	36
Ciprofloxacin	≥ 4	1	0	0	0	0	0	0	0
		2	1	2	0	0	1	2	6
Nalidixic acid	≥ 32	1	0	0	0	0	2	0	2
		2	2	2	2	0	1	1	8
Sulfisoxazole	≥ 256	1	6	6	6	6	6	5	35
		2	6	6	6	6	6	6	36
Tetracycline	≥ 16	1	6	6	6	6	6	6	36
		2	6	6	6	6	6	6	36
Trimethoprim/		1	4	4	4	3	3	0	18
sulfamethoxazole	≥ 4/76	2	6	5	2	3	2	1	19
Amoxicillin/		1	3	3	4	0	0	0	10
clavulanic acid	≥ 32/16	2	4	3	5	4	1	0	17

Table 3. Multi-drug resistance and patterns of resistance in MDR EC isolates by collection, cultured from fecal samples of dairy calves of multiple ages on commercial dairy farms

		Calf Age						Combined
Item	Collection	1 wk	2 wks	1 mo	2 mos	4 mos	6 mos	Ages
No. isolates examined	1	6	6	6	6	6	6	36
in each animal class	2	6	6	6	6	6	6	36
Resistant to:								
2 antibiotics	1	0	0	0	0	0	1	1
	2	0	0	0	0	0	0	0
4 to 6 antibiotics	1	1	0	2	3	5	5	16
	2	0	0	0	1	5	4	10
7 to 9 antibiotics	1	2	4	1	3	1	0	11
	2	2	2	1	1	0	1	7
≥10 antibiotics	1	3	2	3	0	0	0	8
	2	4	4	5	4	1	1	19
At least:								
ACSSuT[a]	1	1	1	0	1	0	0	3
	2	2	4	6	3	0	2	17
MDR-AmpC[b]	1	3	3	4	0	0	0	10
	2	4	2	0	2	1	0	9

[a]ACSSuT = resistant to ampicillin, chloramphenicol, streptomycin, sulfisoxazole, and tetracycline.

[b]MDR-AmpC = resistant to ACSSuT plus amoxicillin/clavulanic acid and ceftiofur, and a decreased susceptibility to ceftriaxone (MIC ≥ 2 µg/ml).

Table 4. MDR EC [fecal concentration = FC; CFU (log_{10})/g feces] and pen prevalence [% pens with calf culture positive for MDR EC (% Pens)] in two groups of dairy calves on a commercial dairy farm, sampled two days pre- and post-weaning (by group and combined)

			Pre-weaning		Post-weaning	
Group	No. samples	No. Pens	FC	% Pens	FC	% Pens
1	69	18	3.3	83	3.8	89
2	75	19	3.7	89	2.9	63
Combined	144	37	3.5	86	3.4	76

Table 5. Multi-drug resistance and patterns of resistance in fecal MDR EC isolates cultured from dairy calves. Data combined from two groups of dairy calves on a commercial dairy farm, two days pre- and post-weaning.

	Time	
Item	Pre-wean	Post-wean
No. isolates examined	10	10
No. isolates resistant to:		
0 - 3 antibiotics	0	0
4 or 5 antibiotics	4	6
6 - 10 antibiotics	6	4
Phenotypes		
ACSSuT[a]	3	2
MDR-AmpC[b]	0	1

[a]ACSSuT=resistant to ampicillin, chloramphenicol, streptomycin, sulfisoxazole, and tetracycline.

[b]MDR-AmpC = resistant to ACSSuT plus resistant to amoxicillin/clavulanic acid and ceftiofur and decreased susceptibility to ceftriaxone (MIC > 2 µg/mL).

Farm Origin and Influence on Antimicrobial Susceptibility of MDR EC

In general, farms were similar in regards to susceptibility/resistance to individual antibiotics. The majority of all MDR EC isolates (greater than 80%) were resistant to chloramphenicol, streptomycin, sulfisoxasole, and tetracycline, while approximately half displayed resistance to amoxicillin/clavulanic acid, cefoxitin, ceftiofur, ceftriaxone, gentamicin, kanamycin, naladixic acid, and trimethoprim/sulfisoxasole (Table 6). Multi-drug resistance (2 to 14 antibiotics) was observed in all 192 isolates examined with most (69%) resistant to 8 or more antibiotics (Table 7). The most prevalent resistance phenotypes were ACSSuT and MDR Amp-C, both found in 36% of the isolates. Multi-drug resistance was similar among farms with the exception of Farms A and C, in which fewer ACSSuT and more MDR AmpC phenotypes were observed on Farm A (Table 7).

DISCUSSION

A few years ago, while investigating a suspected outbreak of salmonellosis, we cultured MDR EC from a relatively large number of young dairy calves. Subsequent examination of the literature revealed that the occurrence of MDR EC had been documented in young dairy calves in other regions of the United States (DeFrancesco *et al.*, 2004; Houser *et al.*, 2008) and that this particular *E. coli*, or the maintenance of resistance in this species, was thought to be restricted to very young calves. The prevalence of resistant organisms is typically higher in younger animals (Brophy *et al.*, 1977; Hinton *et al.*, 1985; Zhang *et al.*, 1998; Mathew *et al.*, 1999). This at first would seem counter-intuitive if the development of antimicrobial resistance is related to previous antibiotic therapy. However, young animals are typically more susceptible to disease and receive antibiotics for the treatment or prevention of such diseases. Even so, it would stand to reason that as age increases, exposure to antibiotics would also increase, and therefore the prevalence of resistant isolates would be greater in older animals. However, as this is not the case in dairy cattle (Edrington *et al.*, 2008; Houser *et al.*, 2008; Lundin *et al.*, 2008), researchers have speculated that perhaps this increased resistance in dairy calves is due to their exposure to more antibiotics for medication and/or growth promotion compared to mature cows. Khachatryan and coworkers (2004) reported just the opposite however, in that the resistant *E. coli* demonstrated a greater fitness in the calf intestinal tract environment that was independent of exposure to antimicrobial drugs and that drug use was not required to maintain a high prevalence of this resistant strain of *E. coli*. Others reported that the clustering of MDR EC in calves 2 to 4 weeks of age, on both dairies and calf ranches, suggest there are host-specific factors influencing the emergence of resistance that may not be associated with antibiotic use (Berge *et al.*, 2005). Taken together, this suggests that the development or maintenance of the resistance of *E. coli* in dairy calves is not dependent on exposure to antibiotics, but was an environmental or diet induced phenomenon.

Table 6. Antimicrobial resistance profiles of fecal MDR EC isolates from dairy calves originating from multiple dairy farms upon arrival at a central heifer raising facility. Data represents the number of isolates resistant to the minimum inhibitory concentration (MIC) listed for each antibiotic.

		Farm of Origin				Combined
Item	MIC	A	B	C	D	Ages (%)
No. isolates examined		38	45	69	40	192
Antibiotic						
Amikacin	≥ 64	1	1	1	1	4 (2.1)
Gentamicin	≥ 16	21	30	23	20	94 (49)
Kanamycin	≥ 64	30	32	36	27	125 (65)
Streptomycin	≥ 64	30	34	53	32	149 (78)
Ceftiofur	≥ 8	18	21	22	18	79 (41)
Ceftriaxone	≥ 64	19	23	29	23	94 (49)
Cefoxitin	≥ 32	19	27	22	19	87 (45)
Ampicillin	≥ 32	35	45	61	38	179 (93)
Chloramphenicol	≥ 32	38	45	69	39	191 (99)
Ciprofloxacin	≥ 4	11	17	20	17	65 (34)
Naladixic acid	≥ 32	19	23	32	23	97 (51)
Sulfisoxazole	≥ 256	38	45	68	40	191 (99)
Tetracycline	≥ 16	38	45	68	40	191 (99)
Trimethoprim/sulfamethoxazole	≥ 4/76	18	24	32	23	97 (51)
Amoxicillin/clavulanic acid	≥ 32/16	20	17	25	24	86 (45)

Table 7. Multi-drug resistance and patterns of resistance (number of isolates and percentage in parentheses) in fecal MDR EC isolates cultured from newborn calves, originating from four different dairies, upon arrival at a central heifer raising facility

	Farm of Origin				Across
Item	A	B	C	D	Farms
No. isolates examined	38	45	69	40	192
No. isolates resistant to:					
0 - 3 antibiotics	0	0	1 (1.5)	0	1 (0.5)
4 - 7 antibiotics	11 (29)	8 (18)	30 (43)	9 (23)	58 (30)
8 - 14 antibiotics	27 (71)	37 (82)	38 (55)	31 (78)	133 (69)
Phenotypes					
ACSSuT[a]	10 (26)	16 (36)	28 (41)	15 (38)	69 (36)
MDR-AmpC[b]	17 (45)	19 (42)	18 (26)	15 (38)	69 (36)

[a] ACSSuT=resistant to ampicillin, chloramphenicol, streptomycin, sulfisoxazole, and tetracycline

[b] MDR-AmpC = resistant to ACSSuT plus resistant to amoxicillin/clavulanic acid and ceftiofur and decreased susceptibility to ceftriaxone
(MIC > 2 µg/mL)

Dairy calves experience a number of changes during a relatively short time frame that may explain the age related decrease for this bacterium. Adaptation and eventual weaning from a liquid, milk based diet to a diet composed of hay and grain, and the associated changes in gastrointestinal microflora could explain these age-related changes. Results of the current research demonstrated an age-related change in fecal populations and prevalence of MDR EC in dairy calves as reported by others and discussed above. We did however culture MDR EC from a substantial number of calves at 6 months of age, older that most of the calves examined in previous research. Examination of calves pre- and post-weaning found no significant differences in MDR EC prevalence or populations. Taken together, these results suggest that the disappearance of MDR EC in dairy calves is a gradual process that is not strongly influenced by changing diet or other animal husbandry factors as we originally hypothesized. If these changes were in fact a result of changing diet and maturation of the digestive system, then we would expect to see a more substantial decline prior to six months of age, as diet changes significantly early in age but are very subtle later (4 and 6 months).

Pasteurization of the waste milk used to feed the calves appeared to have slight influence on MDR EC populations in these dairy calves. Both the number of MDR EC positive samples and the concentration of MDR EC were lower in calves fed the NPWM compared to PWM. Significant reductions (90 to 95%) in total bacterial counts as well as for specific pathogens such as *Salmonella* have been reported following pasteurization of waste milk (Stabel *et al.*, 2004; Ruzante *et al.*, 2008). However, milk that is not properly chilled following pasteurization provides a warm environment for rapid bacterial growth, increasing the number of cells as much as 8-fold per hour. Overall bacterial counts in PWM prior to feeding, were reported to range from 500,000 to 100 million CFU/ml, which was not different from 60% of the farms pasteurizing the milk (Ruzante *et al.*, 2008). Possibly the differences that were observed in this research are a result of competitive exclusion as influenced by the pasteurization process. Pasteurization may have reduced the bacterial species that are more able to compete with the MDR EC, thus providing MDR EC a competitive advantage in the calves fed PWM. Some researchers have hypothesized that the presence of MDR EC in calves fed waste milk is due to a selection pressure maintained through the feeding of low concentrations of antibiotics contained in the milk (Berge *et al.*, 2005). Subsequent examination of the waste milk failed to confirm the presence of antibiotics in the milk and led to the conclusion that feeding hospital milk had no observable impact on antibiotic resistance in *E. coli*. In the current research, if antibiotics in the milk were responsible for the MDR EC, then we would expect to see higher levels in calves fed NPWM, assuming the pasteurization process affected antibiotic residues in the milk. On the other hand, if pasteurization had no affect on the antibiotics in the milk, then we would expect to see similar levels among the feeding groups, not the subtle differences we observed.

Possibly the differences we observed were due to some other farm related factor and not pasteurization of the waste milk. This is certainly plausible and a drawback from the experimental design. Unfortunately, conducting research on commercial dairy farms, while providing for "real-world" settings, does have short-comings; in this case the dairyman pasteurizing waste milk was not willing to feed some of the calves on his farm non-pasteurized milk due to health concerns and labor issues. Therefore the next best scenario was to sample calves on different farms, similar in most all aspects, except for pasteurization of the waste milk. While other factors may have influenced the results, the widespread dissemination of MDR EC among dairy calves and similarity of resistance phenotypes, as observed in the first three collections as well as the fourth collection, comparing calves from four different farms, suggests this is unlikely and the differences are likely due to handling of the waste milk.

Contrary to the research of Khachatryan *et al.* (2004), who reported a greater prevalence of SSuT resistance in milk-fed calves, Hinton *et al.* (1984) found that fecal *E. coli* from calves were more likely to develop MDR resistance during and immediately

after weaning from a medicated milk replacer. In our research, inclusion of the MDR EC isolates collected pre- and post-weaning in this discussion confounds the interpretation. The MDR EC isolates cultured from the weaning study were resistant to fewer different antibiotics (11) and displayed two patterns of resistance (ACSSuT and MDR-AmpC) than isolates from younger calves in the first collection. However, in comparing these two groups of isolates, it must be taken into account that they were collected from different farms with different management techniques and at different times of the year.

Results of this research indicate that the persistence of MDR EC in dairy calves is a function of age. Furthermore, the decline in populations and prevalence does not appear to directly correspond to changes in diet and may be a more subtle indication of gastrointestinal maturation or other factors yet to be determined. While *E. coli* is present in mature cows, it is not reported to be MDR, indicating that maternal transfer is not responsible for its presence in calves but some other environmental factor(s). The gradual disappearance with age, suggest diet may be a limiting factor, although if entirely responsible for the presence and/or disappearance of the bacteria then we might expect bigger decreases in its populations when diet is significantly changed, such as at weaning, and not the steady decline we observed when diet was not changed. We hypothesize that the survival and disappearance is simply a matter of the competitive fitness of this species within the developing gastrointestinal microflora of the calf. Results of this research and of others support this conclusion. Berge and colleagues (2005) suggested that in the young calf-gastrointestinal environment, *E. coli* with multiple antibiotic resistance exhibits a higher fitness compared to susceptible *E. coli*. The intestinal microbiota is very different in a young milk fed calf compared to an adult animal, which the MDR EC appear to find more suitable for survival (Khachatryan *et al.*, 2004). This would suggest that the presence of resistance elements may give the MDR EC a survival advantage over susceptible strains in the developing gastrointestinal tract. However, as resistance generally comes at a cost to the bacteria, we suggest that while the gut is undeveloped in terms of bacterial diversity, the MDR EC is able to successfully compete, however as the bacterial flora diversifies and increases in numbers, the MDR EC loses its competitive advantage due, at least in part, to being MDR and is slowly removed from the gastrointestinal tract. Khachatryan and colleagues (2004) presented a similar explanation. Their research suggested a direct benefit of the resistance genes themselves or linkage to other genes that are adaptive in this environment. However, they went on to say that relative absence of a diverse bacterial fauna, due in part to the milk diet, is indicative that the MDR EC compete effectively only when significant competition is lacking and as the animal ages and the gut matures, the resistance becomes a burden and the MDR EC is excluded from the system. Previous research examining MDR *Salmonella* in dairy calves supports this idea. Similar to these results, we found MDR *Salmonella* only in young calves or sick cows, suggesting that its ability to compete within the gastrointestinal tract depends on an immature or disturbed microflora (Edrington *et al.*, 2008).

The impact of this population of MDR EC on overall calf health appears to be minimal if any, however the potential transfer of resistance elements to pathogenic bacteria such as *Salmonella* cannot be ruled out. Research into the origin or transmission source of this bacteria as well as methods to hasten the elimination from the gastrointestinal tract of the calf could theoretically reduce the potential development of MDR pathogenic bacteria, leading to improved calf health and in the long term, improved herd health. Reducing the "load" of pathogenic bacteria in the production setting has significant food safety implications.

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REFERENCES

Berge, A.C.B., E.R. Atwill, and W.M. Sischo. 2005. Animal and farm influences on the dynamics of antibiotic resistance in faecal *Escherichia coli* in young dairy calves. Prev. Vet. Med. 69:25-38.

Berge, A.C.B., D.A. Moore, and W.M. Sischo. 2006. Field trial evaluating the influence of prophylactic and therapeutic antimicrobial administration on antimicrobial resistance of fecal *Escherichia coli* in dairy calves. Appl. Environ. Microbiol. 72:3872-3878.

Brophy, P.O., P.J. Caffrey, and J.D. Collins. 1977. Sensitivity patterns of *Escherichia coli* isolated from calves during and following prophylactic chlortetracycline therapy. Br. Vet. J. 133:340-345.

Clinical and Laboratory Standards Institute. 2005. *Performance Standards for Antimicrobial Susceptibility Testing*: Fifteenth Informational Supplement. CLSI/NCCLS document M100-S15. Wayne, PA.

DeFrancesco, K.A., R.N. Cobbold, D.H. Rice, T.E. Besser, and D.D. Hancock. 2004. Antimicrobial resistance of commensal *Escherichia coli* from dairy cattle associated with recent multi-resistant salmonellosis outbreaks. Vet. Micro. 98:55-61.

Donaldson, S.C., B.A. Straley, N.V. Hegde, A.A. Sawant, C. DebRoy, and B.M. Jayarao. 2006. Molecular epidemiology of ceftiofur-resistant *Escherichia coli* isolates from dairy calves. Appl. Environ. Microbiol. 72:3940-3948.

Edrington, T.S., T.R. Callaway, R.C. Anderson, and D.J. Nisbet. 2008. Prevalence of multi-drug resistant *Salmonella* on commercial dairies utilizing a single heifer raising facility. J. Food Prot. 71:27-34.

Edrington, T.S., B.H. Carter, R.L. Farrow, A. Islas, G.R. Hagevoort, T.H. Friend, T.R. Callaway, R.C. Anderson, and D.J. Nisbet. 2011. Influence of weaning on fecal shedding of pathogenic bacteria in dairy calves. Foodborne Path. Dis. 8:395-401.

Food and Drug Administration. 2000. *National Antimicrobial Resistance Monitoring System Annual Report*. Available: http://www.cdc.gov/narms/annual/2000/tables/table2.htm. Accessed January 5, 2007.

Gellin, G., B.E. Langlois, K.A. Dawson, and D.K. Aaron. 1989. Antibiotic resistance of gram-negative enteric bacteria from pigs in three herds with different histories of antibiotic exposure. Appl. Environ. Microbiol. 55:2287-2292.

Gilliver, M.A., M. Bennett, M. Begon, S.M. Hazel, and C.A. Hart. 1999. Antibiotic resistance found in wild rodents. Nature. 401:233-234.

Hinton, M., A.H. Linton, and A.J. Hedges. 1985. The ecology of *Escherichia coli* in calves reared as dairy-cow replacements. J. Appl. Bacteriol. 58:131-138.

Hinton, M., P.D. Rixson, V. Allen, and A.H. Linton. 1984. The persistence of drug resistant *Escherichia coli* strains in the majority faecal flora of calves. J. Appl. Bacteriol. 58:131-138.

Houser, B.A., S.C. Donaldson, R. Padte, A.A. Sawant, C. DebRoy, and B.M. Jayarao. 2008. Assessment of phenotypic and genotypic diversity of *Escherichia coli* shed by healthy lactating dairy cattle. Foodborne Path. Dis. 5:41-51.

Howe, K., and A.H. Linton. 1976. A longitudinal study of *Escherichia coli* in cows and calves with special reference to the distribution of O-antigen types and antibiotic resistance. J. Appl. Bacteriol. 40:331-340.

Hoyle, D.V., H.I. Knight, D.J. Shaw, K. Hillman, M.C. Pearce, J.C. Low, G.J. Gunn, and M.E. Woolhouse. 2004. Acquisition and epidemiology of antimicrobial-resistant *Escherichia coli* in a cohort of newborn calves. J. Antimicrob. Chemother. 53:867-871.

Kaneene, J.B., L.D. Warnick, C.A. Bolin, R.J. Erskine, K. May, and R.A. Miller. 2008. Changes in tetracycline susceptibility of enteric bacteria following switching to nonmedicated milk replacer for dairy calves. J. Clin. Microbiol. 46:1968-1977.

Khachatryan, A.R., D.D. Hancock, T.E. Besser, and D.R. Call. 2004. Role of calf-adapted *Escherichia coli* in maintenance of antimicrobial drug resistance in dairy calves. Appl. Environ. Microbiol. 70:752-757.

Khachatryan, A.R., T.E. Besser, D.D. Hancock, and D.R. Call. 2006a. Use of a nonmedicated dietary supplement correlates with increased prevalence of streptomycin-sulfa-tetracycline-resistant *Escherichia coli* on a dairy farm. Appl. Environ. Micro-

biol. 72:4583-4588.

Khachatryan, A.R., D.D. Hancock, T.E. Besser, and D.R. Call. 2006b. Antimicrobial drug resistance genes do not convey a secondary fitness advantage to calf-adapted *Escherichia coli*. Appl. Environ. Microbiol. 72:443-448.

Lundin, J.I., D.A. Dargatz, B.A. Wagner, J.E. Lombard, A.E. Hill, S.R. Ladely, and P.J. Fedorka-Cray. 2008. Antimicrobial drug resistance of fecal *Escherichia coli* and *Salmonella* spp. isolates from United States dairy cows. Foodborne Path. Dis. 5:7-19.

Martel, J.L., and M. Coudert. 1993. Bacterial resistance monitoring in animals: the French national experiences of surveillance schemes. Vet. Microbiol. 35:321-338.

Mathew, A.G., A.M. Saxton, W.G. Upchurch, and S.E. Chattin. 1999. Multiple antibiotic resistance patterns of *Escherichia coli* isolates from swine farms. Appl. Environ. Microbiol. 65:2770-2772.

National Academy of Science. 1999. The use of drugs in food animals: benefits and risks. National Academy Press, Washington, D.C.

O'Brien, T.F. 2002. Emergence, spread, and environmental effect of antimicrobial resistance: how use of an antimicrobial anywhere can increase resistance to any antimicrobial anywhere else. Clin. Infect. Dis. 34(Suppl. 3):S78-S84.

Orden, J.A., J.A. Ruiz-Santa-Quiteria, S. Garcia, D. Cid, and R. de la Fuenta. 2000. In vitro susceptibility of *Escherichia coli* strains isolated from diarrhoeic dairy calves to 15 antimicrobial agents. J. Vet. Med. B 47:329-335.

Ruzante, J.M., I.A. Gardner, J.S. Cullor, W.L. Smith, J.H. Kirk, and J.M. Adaska. 2008. Isolation of *Mycobacterium avium* subsp. *paratuberculosis* from waste milk delivered to California calf ranches. Foodborne Path. Dis. 5:681-686.

Shoemaker, N.B., H. Vlamakis, K. Hayes, and A.A. Salyers. 2001. Evidence for extensive resistance gene transfer among *Bacteroides* spp. and among *Bacteroides* and other genera in the human colon. Appl. Environ. Microbiol. 67:561-568.

Schmieger, H., and P. Schicklmaier. 1999. Transduction of multiple drug resistance of *Salmonella enterica* serovar Typhimurium DT 104. FEMS Microbiol. Lett. 170:251-256.

Smith, H.W. 1975. Persistence of tetracycline resistance in pig *E. coli*. Nature. 258: 628-630.

Sogaard, H. 1973. Incidence of drug resistance and transmissible R factors in strains of *E. coli* isolated from faeces of healthy pigs. Acta Vet. Scand. 14:381-391.

Stabel, J.R., S. Hurd, L. Calvente, and R.F. Rosenbusch. 2004. Destruction of *Mycobacterium paratuberculosis*, *Salmonella* spp., and *Mycoplasma* spp. in raw milk by a commercial on-farm high-temperature, short-time pasteurizer. J. Dairy Sci. 87:2177-2183.

Summers, A.O. 2002. Generally overlooked fundamentals of bacterial genetics and ecology. Clin. Inf. Dis. 34:S85-92.

Van den Bogaard, A.E., and E.E. Stobberingh. 2000. Epidemiology of resistance to antibiotics. Links between animals and humans. Int. J. Antimicrob. Agents. 14:327-335.

Weirup, M., K. Larsson, P. Holtenius, S.O. Jacobsson, and I. Meansson. 1975. The effect of antibiotic supplementation on antibiotic resistance, transferable antibiotic resistance, morbidity, and growth in calves. Nord. Vet. Med. 27:253-265.

Werckenthin, C., S. Seidl, J. Riedl, E. Kiossis, G. Wolf, R. Stolla, and Q.R. Kaaden. 2002. *Escherichia coli* isolates from young calves in Bavaria: in vitro susceptibilities to 14 anti-microbial agents. J. Vet. Med. B 49:61-65.

Winokur, P.L., D.L. Vonstein, L.J. Hoffman, E.K. Uhlenhopp, and G.V. Doern. 2001. Evidence for transfer of CMY-2 AmpC beta-lactamase plasmids between *Escherichia coli* and *Salmonella* isolates from food animals and humans. Antimicrob. Agents Chemother. 45:2716-2722.

Zhang, X.L., F. Wang, D.M. Zhu, S. Wu, P.C. Wu, Y.D. Chen, Y.Q. Wang, and L Zhou. 1998. The carriage of *Escherichia coli* resistant to antibiotics in healthy populations in Shanghai. Biomed. Environ. Sci. 11:314-320.